Myristoylated alanine rich C kinase substrate/Activated Cdc42-associated kinase 1 regulates cortactin to promote neutrophil elastase-induced mucin secretion in airway epithelial cells

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Research Article

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Abstract

Purpose

Mucus secretion is excessively increased in airway epithelial cells in pathological states. This process is related to the cytoskeleton and the increase in exocytosis sites, but the movement of secreted molecules and how secretion increases remain unclear. In this study, we examined the potential role of myristoylated alanine rich C kinase substrate (MARCKS) and the cortical actin-binding protein cortactin in airway mucin secretion. Also we investigated the effect of activated Cdc42-associated kinase 1 (ACK1) in this process.

Methods

Human airway epithelial cells were treated with neutrophil elastase (NE) after treatment with siRNA to specifically knock down MARCKS, ACK1 and cortactin expression. The expression and localization of cortactin and MARCKS were observed by western blotting and immunofluorescence, and the phosphorylated forms of MARCKS, cortactin and ACK1 were detected. The interaction of cortactin and ACK1 was analyzed by coimmunoprecipitation. MUC5AC protein expression was measured by ELISAs.

Results

Phosphorylated cortactin was highly expressed, mainly at the cell membrane, after NE stimulation, and phosphorylated MARCKS was mainly expressed in the cytoplasm. Coimmunoprecipitation revealed that ACK1 and cortactin interacted with each other. Knockdown of MARCKS suppressed phosphorylation of cortactin, while cortactin siRNA had no significant effect on MARCKS activation. Knockdown of MARCKS, cortactin and ACK1 by siRNA attenuated the phosphorylation of cortactin and reduced MUC5AC secretion.

Conclusion

These results suggest that both cortactin and MARCKS are involved in MUC5AC secretion by increasing F-actin polymerization and translocation and that MARCKs and ACK1 play an important role in the activation of cortactin.

Introduction

Mucus secretion is excessively increased in airway epithelial cells in pathological states. Mucus hypersecretion is a well-known symptom in respiratory diseases, either in lung infection or inflammation [1,2]. Overproduction of mucin impedes the lumen of the respiratory tract and limits optimal airflow. Furthermore, excessive mucins cause ciliary dysfunction that decreases respiratory clearance, potentially
triggering various inflammatory pathways. Mucin production, including mucin gene transcription, protein translation and secretion, has been studied extensively in recent years. In some pathological conditions, rapid mucin secretion is observed. The secretion process is related to the cytoskeleton and the increase in exocytosis sites, but the movement of secretion molecules and how exocytosis sites increase remain unclear.

The cell cortex is a transparent area approximately 3–5 µm thick that is underneath and closely connected with the plasma membrane. The cortex is formed by actin microfilament binding protein and represents a dense cortical layer of actin filaments, and the fluidity of membrane proteins is restricted to some extent. Since the cell cortex structure is quite dense, the pore size is only 0.1 nm, and all organelles and macromolecules, including secretory granules, cannot pass through it. Therefore, how secretory granules pass through this dense structure and the molecular mechanism of the directional displacement of secretory granules guided by F-actin remain unclear. The cell cortex together with its associated membrane is the most active structure in most of the polar distributed cells. Molecular signaling is transduced successively in the cortex. Cortactin is a cortical actin crosslinking protein that exists widely in polarized epithelial cells and fibroblasts in cortical cells and in aortic smooth muscle cells. Cortactin was named cell cortical actin binding peptide (cortical actin-binding protein, cortactin) for its special subcellular localization. Cortactin may interact with the cytoskeleton and affect the movement (fluidity) of the membrane; it can regulate actin dynamics and participate in exocytosis, cell permeability, cell migration, and invasion. However, the role of cortactin in mucin secretion has not been fully elucidated. Our previous study showed that cortactin is involved in the mucus secretion process by affecting F-actin polarization.

Here, we investigated the interaction of myristoylated alanine rich C kinase substrate (MARCKS) and cortactin in regulating mucin secretion in human airway epithelial cells. We used neutrophil elastase (NE) as an inducer of mucus secretion. MARCKS and cortactin were shown to affect the rearrangement of actin cytoskeleton and mucin secretion. Cortactin and F-actin colocalized at the cell membrane. MARCKS and activated Cdc42-associated kinase 1 (ACK1) knockdown attenuated phosphorylation of cortactin and reduced mucin(MUC)5AC secretion in airway epithelial cells cultured in vitro, whereas cortactin siRNA did not affect the activation of MARCKS. Further study showed that cortactin interacted with ACK1. Our study establishes a unique role for MARCKS and ACK1 in regulating mucin secretion by interacting with cortactin.

Materials And Methods

Materials

DMEM/Ham’s F12 medium, HEPES, fetal bovine serum (FBS), anti-β-actin monoclonal antibody, Lipofectamine 2000 Reagent and Opti-MEM reduced serum medium were purchased from Invitrogen (San Diego, CA). Protein A/G PLUS-agarose immunoprecipitation reagent and FITC-conjugated fluorescent secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).
Rabbit anti-MARCKs antibody was purchased from Cell Signaling Technology (MA, USA). Rabbit anti-p-cortactin antibody and rabbit anti-p-MARCKs antibody were purchased from Affinity (OH, USA). Mouse anti-cortactin antibody (ab33333) was purchased from Abcam (Cambridge, United Kingdom). HRP-conjugated goat anti-mouse antibody and HRP-conjugated goat anti-rabbit antibody were purchased from Boster (Wuhan, China), and neutrophil elastase (NE) was purchased from Merck (Kenilworth NJ, USA).

**Tissues**

Human lung tissues were obtained from surgical specimens resected at the Department of Thoracic Surgery of the First Affiliated Hospital (Hainan Medical University) between 2017 and 2019. The experiments were approved by the institutional ethics committee, and informed consent was obtained prior to sampling. The subjects consisted of 12 patients (5 men, 7 women) aged 36–75 years. At the time of operation, 5 patients were suffering from mild to moderate COPD or asthma, and 7 patients had no underlying airway chronic inflammatory diseases. The tissues were examined by hematoxylin and eosin (HE) staining to confirm that they were noncancerous. Human bronchi for immunohistochemistry were frozen immediately in liquid nitrogen and stored at -80°C for further use.

**Cell culture and treatment**

Human airway epithelial cells (16HBE14o-) were cultured and separately plated in a six-well plate at $5 \times 10^5 - 6 \times 10^5$ cells per well and cultured in 2 mL of DMEM/Ham's F12 medium containing 10% fetal bovine serum. The cells were incubated at 37°C in a 5% CO$_2$ humidified incubator. The medium was changed to growth factor-free medium before experimental assays were conducted. The cells were treated with different concentrations of neutrophil elastase (NE) (0, 25, 50, 100 ng/ml) for 24 h or transfected with siRNA. Cell supernatants and lysates were collected and assayed as described below.

**SiRNA transfection**

SiRNA sequences were designed and verified by qPCR and synthesized by Biofavor Biotech (Wuhan, China). Cells were transfected with cortactin (CTTN) siRNA, MARCKS siRNA, or ACK1 siRNA to investigate the role of cortactin, MARCKS or ACK1. The cells were incubated at a density of $1.5 \times 10^5$ cells/ml in 24-well plates and cultured with 0.45 ml of serumfree Opti-MEM in each well. Lipofectamine™ 2000 (5 µl) was diluted with 100 µl of serumfree medium to reach a final volume of 100 µl. Aliquots (20 µM) of MARCKS siRNA, ACK1 siRNA or control siRNA were diluted with 100 µl of serumfree medium. Subsequently, the diluted siRNA and transfection reagent were mixed and incubated for an additional 20 min at room temperature. Two hundred microliters of transfection mixture was added dropwise to each well and vortexed gently for 10 sec, followed by incubation at room temperature for 6 h. After careful removal of the supernatant, the cells were washed 3 times with PBS and subsequently incubated with fresh Opti-MEM containing 10% fetal bovine serum for 24 h prior to further treatments. Sequence for siRNA CTTN (cortactin): forward 5'-GGAGAAAUUGCAGCUGCAUTT-3', reverse 5'-UUGUCGAUACCUGAUUUUGCTT-3'; siRNA MARCKS: forward 5'-GGAACGGACAGGAGAAUGTT-3', reverse 5'-
CAUUCUCUGUCCGUUCGCTT-3'; siRNA ACK1: forward 5'-GCAAGUCCUGGAUGAGUAATT-3', reverse 5'-UUACUCAUCCACGACUUGCTT-3'.

**ELISAs for MUC5AC protein**

MUC5AC protein levels in the supernatant and cell lysates were measured by using a Human MUC5AC (Mucin 5 Subtype AC) ELISA Kit (Elabscience, Wuhan, China). Supernatants or cell lysates were prepared with PBS at multiple dilutions. The enzyme plates were coated with 100 µl of each sample and incubated at 37 °C for 90 min. The plates were washed 5 times with PBS, 100 µl of biotinylated antibody working solution was added to each well (prepared within 20 min before use), and the plates were incubated at 37 °C for 1 h and then washed 5 times, shaken and patted on absorbent paper until dry. Then, 100 µl of enzyme binding working solution was added to each well (prepared within 20 min before use, placed in the dark), and the plates were incubated at 37 °C for 30 min and then washed 5 times. Then, 100 µl of TMB was added and incubated at 37 °C in the dark for 15 min. Then, 50 µl of stop solution was added to stop the reaction. The optical density (OD) value was read at 450 nm with an enzyme reader (Flexstation3, Molecular Devices).

**Immunohistochemistry**

The localization of cortactin was examined by using immunohistochemical staining with an antibody against cortactin in frozen sections of human bronchi. Six-micrometer-thick sections were cut and mounted on SuperFrost Plus slides. Tissue slides were incubated with rabbit anti-cortactin polyclonal antibody, anti-phospho-cortactin antibody or MARCKS antibody (all in 1:500 dilution) at room temperature for 1 h and then incubated with goat-anti mouse antibody or goat-anti rabbit antibody (1:500 dilution) for 30 min and streptavidin-peroxidase complex (1:100 dilution) for another 45 min. Then, 3,3'-diaminobenzidine (DAB) was used as a chromogen. Phosphate-buffered saline (PBS) was used as a negative control. Epithelial cells staining positively for cortactin were counted and expressed as a percentage of the total number of epithelial cells.

**Coimmunofluorescence for cortactin and MARCKS, p-cortactin and actin**

Cells were fixed with 4% paraformaldehyde and then permeabilized with 0.1% Triton X-100 in PBS for 30 min. Then, the cells were rinsed, blocked in 1% BSA plus 1% normal goat serum and incubated with mouse anti-phospho-cortactin (Tyr421) polyclonal antibody (1:100, Affinity) and mouse anti-F-actin monoclonal antibody (1:100, Abcam) in sequence overnight at 4°C. After three 3-min washes in PBST, slides were incubated with Cy3-conjugated fluorescent goat anti-mouse IgG antibody (1:100, Boster) and FITC-conjugated fluorescent goat anti-rabbit IgG antibody (1:100, Boster) in sequence for 1 h at 37°C. After PBST washes for 3 min, the slides were incubated for 1 h at 37°C. For coimmunofluorescence for cortactin and MARCKS, cells were incubated with mouse anti-cortactin monoclonal antibody (1:100, Abcam) and rabbit anti-MARCKS polyclonal antibody (100, CST), respectively, and then incubated with Cy3-conjugated fluorescent goat anti-mouse IgG antibody (1:100) and FITC-conjugated fluorescent goat
anti-rabbit IgG antibody (1:100) for 1 h at 37°C. The samples were examined with an Olympus BX53 fluorescence microscope.

**Immunofluorescence for p-MARCKs and p-ACK1**

Cells were fixed with 4% paraformaldehyde and then permeabilized with 0.1% Triton X-100 in PBS for 30 min. Then, the cells were rinsed, blocked in 1% BSA plus 1% normal goat serum and incubated with rabbit polyclonal to MARCKs (phospho S162) antibody (1:100, Abcam) or rabbit polyclonal to ACK1 (phospho Y284) (1:100, Abcam) antibody overnight at 4°C. After three 3-min washes in PBST, slides were incubated with Cy3-conjugated fluorescent secondary antibody (1:100) for 1 h at 37°C. After the staining procedure, the immunofluorescence was visualized using an Olympus BX53 fluorescence microscope.

**Western blots for detecting cortactin, MARCKS, p-cortactin, p-MARCKs, and pACK1 protein**

Cells were lysed on ice in lysis buffer containing PMSF before centrifugation. Total protein was determined by a BCA Protein Assay Kit (Beyotime, Beijing, China). Forty micrograms of protein from each group was separated by 12% SDS-PAGE (Beyotime) and transferred to a PVDF membrane (0.45 µm, Millipore). After the PVDF membrane was blocked with TBST with 5% skim milk powder (phosphorylated protein was blocked with 1% BSA), it was probed with anti-MARCKS antibody (1:800, Cell Signaling), anti-p-MARCKS antibody (1:800, Affinity), anti-cortactin antibody (1:1000, Abcam), anti-pTyr421-cortactin antibody (1:1000, Affinity), anti-p-ACK1 antibody (1:1000, Affinity), or anti-GAPDH antibody (1:1000, Goodhere, Hangzhou, China) as a loading control at 4°C overnight and then incubated with HRP-conjugated secondary antibodies (1:1000, Boster, Wuhan, China) at 37°C for 2 h. The protein bands were visualized by enhanced chemiluminescence using BandScan.

**Immunoprecipitation**

The hCTTN-N-Myc-OE and ACK1-N-EGFP-OE plasmids were constructed by using the seamless cloning strategy. Primers for cortactin (hCTTN): 5’-TTGGTACCGAGCTGCacccatggaacaaaaactcatctcag-3’, 5’-GATATCTGCAGCTGACAatgaccccaggcactccgggacccagg-3’. ACK1: 5’-GACGAGCTGTACACGcgtcaacgggactccgggacccagg-3’, 5’-GTCGACTGCAGATTCgtctcagcgggactccgggactccgggacccagg-3’. After cotransfection, the cells were lysed on ice for 10–15 min and collected after full lysis. Five hundred micrograms of protein was collected and brought to a volume of 500 µl with precooled PBS. (The remaining protein was used as a western blot input control). Agarose protein A + G beads were mixed, washed twice with precooled PBS at 3000 rpm for 5 min and then prepared to a 50% concentration with precooled PBS. Agarose protein A + G was divided into two parts, one for removing nonspecific binding and the other for binding antibody. The samples were pretreated with agarose A + G, and 50 µl of agarose A + G (50%) was added to each tube (to eliminate nonspecific binding and reduce the background). The samples were slowly rotated at 4°C for 2 h and then 3000 rpm for 5 min to remove protein A + G beads. The supernatant was collected. Then, 4 µg Myc antibody was added to 500 µl of total protein and reacted with the target protein. The mixture was shaken slowly at 4°C overnight. Fifty microliters of 50% agarose protein A + G (30 µl/tube) was added, and the reaction was performed at 4 °C for 3–6 h. Then, the
mixture was vortexed at 3000 rpm for 5 min. The precipitates were washed with precooled PBS 3 times. The precipitate was suspended in 50 µl of loading buffer, boiled for 5 min, placed on ice immediately, cooled to room temperature, and vortexed at 12000 rpm for 10 min, and 30 µl was used for loading. GFP and Myc were detected by western blotting with input as a control.

Statistics

All data are presented as the mean ± SEM. Statistical analyses were performed with SPSS 23 software (SPSS, Inc.). All experiments were performed with at least 3 cell cultures. Student’s t test or one-way ANOVA (followed by LSD analysis) was used to compare two or more groups. \( P < 0.05 \) was considered to indicate a statistically significant difference.

Results

Cortactin and MARCKS expression in human bronchial epithelium and 16HBE14o- cells

We chose specimens from individuals that had chronic inflammation, including chronic bronchitis, COPD or asthma, and used normal bronchus as a control to detect the expression of cortactin and MARCKS by immunohistochemistry. Cortactin and phosphorylated cortactin were expressed both in the normal and inflammation groups, and phosphorylated cortactin was obviously highly expressed in the airway epithelium with chronic inflammation and was mainly located in the subepithelial membrane (Fig. 1A-D, Fig. 1I). MARCKS was detected at the brush border of epithelial cells in both the noninflammation and inflammation groups, and phosphorylation of MARCKS was significantly greater in epithelium with inflammation (Fig. 1E-H, Fig. 1J).

Since both MARCKS and cortactin have been individually shown to regulate F-actin relocation, we investigated whether both proteins localized in airway epithelial cells. We treated 16HBE14o- cells with NE. The colocalization of cortactin and MARCKS was assayed by coimmunofluorescence. Immunofluorescence assays showed coexpression of MARCKS and cortactin in both the normal control cells and the NE-stimulated cells. In the cells without stimulation, MARCKS was mainly expressed at the cell membrane. After NE stimulation, the overall fluorescence intensity did not show a significant change, but increased MARCKS was translocated from the cell membrane to the cytosol, and more cortactin was detected in the cell membrane, showing a polar distribution (Fig. 2).

NE promotes MARCKS phosphorylation, cortactin phosphorylation, actin polarization, and ACK1 phosphorylation

The 16HBE14o- cells were incubated with different doses of NE (0, 25, 50, 100 ng/ml). Western blot analysis revealed that total cortactin and MARCKS protein levels did not show significant changes in
cells. Phosphorylated cortactin (pTyr421-cortactin) and p-MARCKS protein levels were increased with increasing NE concentration (Fig. 3). Immunofluorescence assays indicated that NE promotes the rearrangement of actin and cortactin phosphorylation, mainly at the cell membrane, and F-actin colocalizes with p-cortactin (Fig. 4). NE also promoted MARCKS phosphorylation and induced MARCKS translocation from the plasma membrane to the cytosol (Fig. 5).

MARCKS was reported to be an activator of ACK1. To investigate whether ACK1 participates in NE-induced responses, we assayed the expression of phosphorylated ACK1 (p-ACK1) protein by immunofluorescence. The results showed that the immunofluorescence intensity of p-ACK1 increased after NE treatment in a dose-dependent manner, indicating that ACK1 may have a potential role in this process (Fig. 6).

**MARCKS and ACK1 knockdown decreases cortactin phosphorylation and translocation, while cortactin was not affected by the phosphorylation of MARCKS**

To assess the effect of MARCKS and ACK1 on cortactin, we transfected cells with MARCKS siRNA, ACK1 siRNA and cortactin siRNA. The expression of p-cortactin was determined by western blotting and immunofluorescence. MARCKS and ACK1 knockdown reduced the phosphorylation of cortactin and decreased F-actin arrangement (Fig. 7A, 7B, 7C, and 7D), indicating the important effect of MARCKS on ACK1 in NE-modulated cytoskeletal reorganization. To investigate the effect of cortactin on MARCKS expression, we assayed the expression of p-MARCKS after cortactin siRNA transfection. However, the phosphorylation of MARCKS did not show a significant change compared with that of the control group (Fig. 8).

**MARCKs regulates ACK1 (activated Cdc42-associated kinase 1) activity**

To further explore whether ACK1 is the kinase responsible for cortactin tyrosine phosphorylation, we transfected cells with ACK1 siRNA or MARCKS siRNA, and phosphorylation of ACK1 and cortactin was assayed by western blots and immunofluorescence. The results showed that NE induced ACK1 Tyr284 expression, and knockdown of MARCKS by siRNA decreased pTyr284 ACK1 expression (Fig. 9A, 9B, and 9C), indicating that MARCKS may affect cortactin activation by regulating ACK1 activity.

**ACK1 binds to cortactin and is responsible for cortactin tyrosine phosphorylation and translocation**

To determine the role of ACK1 in the activation of cortactin, we cotransfected cells with GFP-tagged ACK1 (to enhance ACK1 detection) and Myc-tagged cortactin. The binding of ACK1 and cortactin was assayed by coimmunoprecipitation. GFP-ACK1 precipitated with endogenous cortactin (Fig. 10), suggesting that the two proteins interact with each other. The immunoprecipitation was specific, since beads lacking anti-cortactin antibody did not precipitate GFP-ACK1 (Fig. 10).
Knockdown of MARCKS and cortactin by siRNA inhibits MUC5AC secretion

To determine the role of MARCKS and cortactin in MUC5AC secretion, we transfected cells with cortactin siRNA and MARCKS siRNA before NE stimulation, and MUC5AC protein expression was detected by ELISAs. The results showed that MUC5AC protein expression was attenuated in both the cortactin siRNA- and MARCKS siRNA-transfected groups. These results showed that both cortactin and MARCKS are involved in MUC5AC secretion, with a decrease in F-actin arrangement and translocation (Fig. 7, Fig. 11).

Discussion

In this study, we showed that NE promotes F-actin rearrangement and MUC5AC production in 16HBE14o-cells, with phosphorylation of cortactin and MARCKS. Inhibition of both cortactin and MARCKS by siRNA silencing effectively suppressed F-actin translocation and MUC5AC secretion. Importantly, NE also induced p-ACK1 expression in a dose-dependent manner. SiRNA-mediated knockdown of MARCKS or ACK1 attenuated p-ACK1 expression and cortactin Tyr421 phosphorylation and disrupted actin fiber rearrangement. Furthermore, we used coimmunoprecipitation to demonstrate that cortactin interacted with ACK1. Thus, this study provided strong evidence that both cortactin and MARCKS are critical modulators of actin filament formation and mucin production in airway epithelial cells. Both MARCKS and ACK1 may be important regulators of the activation of cortactin.

Mucus hypersecretion is a prominent manifestation during chronic inflammatory diseases and infectious diseases, including COPD, asthma, pneumonia and even SARS-CoV-2 infection [7,8]. The hypersecreted mucus hinders drug delivery to affected sites in the airway, since most drugs cannot penetrate the thick mucus wall or are trapped by mucus and can only be cleared through the physiological mucociliary clearance system. Excessive mucin is also conducive to airflow obstruction, bacterial infection and colonization [9,10]. Therefore, targeting mucus hypersecretion is an effective treatment strategy to improve the mortality and morbidity of these airway hypersecretory diseases [11].

Mucins are secreted at a low rate at baseline and a high rate after stimulation, and both rates are regulated by extracellular ligands and second messengers [12]. Various secretory factors can induce mucin synthesis and secretion, such as adrenergic agonists, bacterial products, cytokines, and proteinases [13–15]. The regulation of airway mucus secretion was reported to be mediated by the phosphorylation of myristoylated alanine-rich C kinase substrate (MARCKS), which is shifted from the membrane to the cytoplasm and stabilizes adhesion to the granular membrane after dephosphorylation [16,17]. PMA [18], protein kinase C (PKC) [19], heat shock protein 70 [20], calmodulin, phosphatidylinositol-4,5-bisphosphate (PIP2), and many other kinases can increase mucin secretion through MARCKS activation [21,22]. MARCKS is a highly conserved membrane-associated protein involved in the structural modulation of the actin cytoskeleton [23], chemotaxis, cell adhesion, phagocytosis, and exocytosis. This molecule has an N-terminal myristoylated domain for membrane binding, a highly conserved MARCKS homology 2
(MH2) domain, and an effector domain (which is the phosphorylation site) [24–26]. MARCKS is combined with F-actin or myosin and links mucin particles to cytoskeletal proteins such as F-actin [27]. F-actin moves the mucin particles to the cell membrane and forms a soluble N-ethyl maleimide sensitive factor attachment protein receptor complex through exocytosis, mediates the fusion of the secretory vesicle membrane and the cell membrane, and then releases the intracellular mucin particles out of the cell [28,29]. There are three postulated roles for MARCKS in regulated exocytosis: it tethers cortical actin filaments to the plasma membrane and/or secretory granules and/or it sequesters PIP2. However, studies also suggested that MARCKS likely does not tether actin filaments to secretory granules [30].

We report here that NE stimulated MARCKS phosphorylation and F-actin arrangement in cell plasma, with increasing production of MUC5AC, consistent with previous findings [19]. Knockdown of MARCKs by siRNA interference could disturb the arrangement of F-actin and decrease MUC5AC secretion, and the phosphorylation and translocation of cortactin were also reduced, indicating that MARCKS could affect the function of cortactin. Since both MARCKS and cortactin are critical for the regulation of the actin cytoskeleton, exactly how MARCKS and cortactin interact to regulate the exocytotic release of mucin in airways has not been extensively elucidated; thus, in this study, we further investigated the interaction between these molecules.

Cortactin is a cortical binding protein that is widely involved in cell migration and invadopodia [31]. Most studies of cortactin are focused on tumor progression and invasion; cortactin also facilitates the neurosecretory process, and it can regulate exocytosis via a mechanism independent of actin polymerization in chromaffin cells [32]. Recent studies have reported that cortactin is involved in many functional changes, including inflammatory responses, bronchoconstriction, alveolar disruption with increased permeability, and airway secretion [33,34]. This molecule is associated with several important lung diseases, including COPD, asthma, aspirin-exacerbated respiratory syndrome [35], and acute lung injury [36]. Cortactin may participate in the development of COPD, particularly that induced by cigarette smoke. Cigarette smoke or e-cigarette condensate could increase ROS generation and lung endothelial cell apoptosis through rearrangement of the actin cytoskeleton and increased cortactin tyrosine phosphorylation, and CTTN siRNA or SH3 domain blocking peptide could decrease lung endothelial cell apoptosis [37]. Cortactin deficiency protected against hemin-induced injury in human lung microvascular endothelial cells both in vivo and in vitro by decreasing p38/HSP27 expression and ROS production and improving cell barrier function [38]. Phosphorylated cortactin Tyr421 facilitates the accumulation of actin-regulatory protein profilin-1 at cell edges, promotes actin polymerization and cell movement, and plays a pivotal role in inducing smooth muscle contraction and airway hyperresponsiveness in asthma [34,39,40]. However, whether cortactin participates in airway mucin secretion has not been fully elucidated.

Our previous study showed that cortactin is involved in sheer stress-induced mucin secretion by regulating F-actin polarization [41]. In this study, neutrophil elastase induced MUC5AC secretion and production, with phosphorylation of cortactin and arrangement of F-actin. Then, we transfected cells with cortactin (CTTN) siRNA. MUC5AC secretion was reduced, and the location of F-actin around the cell
membrane was decreased. SiRNA-mediated knockdown of both MARCKS and CTTN resulted in stronger attenuation of MUC5AC secretion and F-actin arrangement than that of CTTN siRNA-transfected cells or MARCKS siRNA-transfected cells alone, indicating that both MARCKS and cortactin could promote mucin secretion by stabilizing F-actin polymerization. We also found that cortactin phosphorylation can be restrained by MARCKS siRNA, which is consistent with a previous report that MARCKS could influence the activation and translocation of cortactin [42].

MARCKS can regulate the activity of several small proteins, such as GTP Rac1 and Cdc42 [42]. Activated Cdc42-associated kinase 1 (ACK1/TNK2) was initially identified as a nonreceptor tyrosine kinase that specifically binds to the GTP-bound form of Cdc42; it shuttles between the cytosol and the nucleus to rapidly transduce extracellular signals from tyrosine kinases to intracellular effectors. Recent data have shown that it is involved in the development of multiple malignancies [43], inflammation and autoimmune diseases [44]. Activated Cdc42 leads to actin polymerization into filopodia, and the downstream effectors of Cdc42 and ACK are also activated. Studies have identified class 1A PI3K (PI3Ks) as a novel ACK1 binding partner that is critical for PDGF-dependent actin rearrangement, indicating that ACK1 may also participate in actin arrangement [45–47]. Additionally, ACK1 can directly bind and phosphorylate the Arp2/3 regulatory protein cortactin, contributing to ligand-mediated EGFR internalization [48]. Here, we further investigated whether MARCKS could also regulate ACK1. We found that NE induced ACK1 (Y284) activation in cells. MARCKS siRNA but not CTTN siRNA inhibited the expression of pACK1, whereas ACK1 siRNA inhibited p-cortactin expression. Coimmunoprecipitation showed that cortactin and ACK1 combined, suggesting that MARCKS could regulate ACK1 and ACK1 combined with and then activated cortactin to promote the polarization of F-actin.

Cortactin may influence mucin secretion in the following ways. First, cortactin is involved in the processes of membrane remodeling; it strengthens the stability of F-actin and modulates filament organization [49,50], and it can reduce the depolymerization of F-actin by promoting and stabilizing the branched chained F-actin networks [51]. Second, cortactin contributes to the Ca^{2+}-dependent formation of F-actin; it regulates exocytotic events depending on the availability of monomeric actin and its phosphorylation by ERK1/2 and Src kinases but independent of actin polymerization [32]. Third, this molecule may promote coated vesicle formation and mucin granule release [52]. Fourth, cortactin may be involved in mucin gene expression by ERK signaling [53], which is an interesting hypothesis that needs further investigation.

**Conclusion**

- In conclusion, our study described the interaction of cortactin and MARCKS in airway inflammation. Both molecules are membrane-tracking proteins that can modulate cytoskeletal signaling and promote mucin secretion under NE stimulation. ACK1 may serve as an intermediator for this interaction. The overall effects of cortactin and MARCKS in airway inflammatory diseases still need further elucidation. Whether the gene polymorphisms of both MARCKs and cortactin affect the
arrangement of F-actin or regulate mucin secretion or even inflammatory factor production should be explored in future investigations.

Declarations

Ethics approval and consent to participate

The tissue study was reviewed and approved by the Ethics Committee of The First Affiliated Hospital of Hainan Medical University [2020 scientific research (No.91)], and informed consent was obtained prior to sampling. Study was carried out in accordance with the Declaration of Helsinki. All methods were carried out in accordance with relevant guidelines and regulations in the Ethics approval.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interest

The authors declare that they have no competing interests.

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Authors' contributions


Acknowledgements

Not applicable.

Author details
References


**Figures**

**Figure 1**

*Expression of cortactin, p-cortactin, MARCKS and p-MARCKS in human airway epithelium.* (A) Cortactin expression in the noninflammation group. (B) p-cortactin in the noninflammation group. (C) cortactin expression in the inflammation group; (D) p-cortactin expression in the inflammation group. (E) MARCKS expression in the noninflammation group. (F) p-MARCKS expression in the noninflammation group. (G) MARCKS expression in the inflammation group. (H) p-MARCKS expression in the inflammation group. (I)
Relative expression of cortactin and p-cortactin in different groups. Data were expressed as mean±SEM, n=3. ns: no significance; *P<0.05. (J) Relative expression of MARCKS and p-MARCKS in different groups. Data were expressed as mean±SEM, n=3. ns: no significance; *P<0.05.

Figure 2
Coexpression of cortactin and MARCKS in 16HBE14o- cells after NE stimulation. (A) The long arrow indicates the membrane location of MARCKS, and the short arrow indicates the polar distribution of cortactin in the cell membrane. Scale bar=20 μm. (B) The intensity of fluorescence of cortactin and MARCKS protein expression in normal control group and NE-treated group. Data were expressed as mean±SD, n=3. ns: no significance.

Figure 3

Cortactin and MARCKS protein expression after NE stimulation. (A) Cells were treated with NE (0, 25, 50, 100 ng/ml), cortactin, phosphorylated (p)-cortactin, MARCKS and p-MARCKS were assayed by western blotting. The group ing of blots were cropped from different parts of the same gel. (B) Relative expression of MARCKS and p-MARCKS in different groups. Data were expressed as mean±SEM, n=3. *P<0.01, compared with the untreated group. (C) Relative expression of cortactin and p-cortactin in different groups. Data were expressed as mean±SEM, n=3. *P<0.01, compared with the untreated group.
Figure 4

Coexpression of p-cortactin and F-actin in 16HBE14o- cells shown by immunofluorescence assays after NE treatment. (A) Cells were treated with different doses of NE (0, 25, 50, 100 ng/ml). Scale bar=50 μm. (B) The intensity of fluorescence of p-cortactin and F-actin protein expression in different NE-treated group. Data were expressed as mean±SEM, n=3. *P<0.01, compared with non-treated group; **P<0.01, compared with 25 ng/ml NE-treated group.
Figure 5

**Immunofluorescence assay of phosphorylated MARCKS (p-MARCKS) after NE treatment.** (A) Cells were treated with different doses of NE (0, 25, 50, 100 ng/ml). Scale bar=50 μm. (B) The intensity of fluorescence of p-MARCKS expression in different NE-treated group. Data were expressed as mean±SEM, n=3. *P<0.01, compared with non-treated group; **P<0.01, compared with 25 ng/ml NE-treated group.
Immunofluorescence assay of phosphorylated ACK1 (p-ACK1) after NE treatment. (A) The 16HBE14o-cells were treated with 0, 25, 50, or 100 ng/ml NE, and red fluorescence indicates p-ACK1. Scale bar=50 μm. (B) The intensity of fluorescence of p-ACK1 expression in different NE-treated group. Data were expressed as mean±SEM, n=3. *P<0.01, compared with non-treated group.
Figure 7

Expression of p-cortactin and F-actin after MARCKS and ACK1 siRNA transfection. (A) p-cortactin expression after MARCKS siRNA transfection. *P<0.01, compared with the untreated group. **P<0.01 compared with the merely NE-treated group. p-Cort: p-cortactin. (B) p-cortactin expression after ACK1 siRNA transfection. *P<0.01, compared with the untreated group. **P<0.01, compared with merely NE-treated group. The grouping of blots were cropped from different parts of the same gel. (C) Coimmunofluorescence assay of p-cortactin and F-actin. Cells were transfected with cortactin siRNA, MARCKS siRNA and ACK1 siRNA, and NC siRNA was used as a control. Colocalization of p-cortactin and F-actin was detected by immunofluorescence. (D) The intensity of fluorescence of p-cortactin and F-actin expression in different siRNA-transfected group. Data were expressed as mean±SEM, n=3. *P<0.01, compared with NC siRNA-transfected group. **P<0.05, compared with NC siRNA-transfected group.
**Figure 8**

**Immunofluorescence assay of p-MARCKS.** Cells were transfected with cortactin siRNA and MARCKS siRNA, and NC siRNA was used as a control. p-MARCKS in each group was detected by immunofluorescence. Scale bar=50 μm. (B) The intensity of fluorescence of p-MARCKS expression in different siRNA-transfected group. Data were expressed as mean±SEM, n=3. *P<0.01, compared with NC siRNA-transfected group.
Figure 9

Expression of p-ACK1 after MARCKS siRNA transfection. (A) Phosphorylated ACK1 protein expression detected by western blots assay after MARCKS siRNA transfection. *P<0.01, compared with the negative control group, **P<0.01, compared with the merely NE-treated group and the NE+NC siRNA-transfected group. The grouping of blots were cropped from different parts of the same gel. (B) Immunofluorescence assay of p-ACK1. Cells were transfected with MARCKS siRNA and ACK1 siRNA, and NC siRNA was used...
as a control. p-ACK1 was detected by immunofluorescence. Scale bar=50μm. (C) The intensity of fluorescence of p-ACK1 expression in different siRNA-transfected group. Data were expressed as mean±SEM, n=3. *P<0.01, compared with NC siRNA-transfected group.

**Figure 10**

**Immunoprecipitation assay of cortactin and ACK1.** Cells were cotransfected with Myc-cortactin and GFP-ACK1 or transfected with Myc-cortactin only. Myc was used to immunoprecipitate the specific ACK1-cortactin complex. Different groups of samples were immunoblotted with rabbit anti-Myc antibody or rabbit anti-GFP antibody. IB: Myc-cortactin approximately 85 kDa; IB: GFP-ACK1 approximately 140 kDa.
Figure 11

Secretion of MUC5AC protein in cell supernatants. (A) MUC5AC protein after NE stimulation. Cells were treated with different concentrations of NE (0, 25, 50, 100 ng/ml), and the secretion of MUC5AC was assayed by ELISAs. \(*P < 0.01\), compared with the untreated group; \(**P < 0.01\), compared with the 25 ng/ml group. (B) MUC5AC protein expression after cortactin and MARCKS knockdown. Cells were treated with 100 ng/ml NE after siRNA transfection. The secretion of MUC5AC was assayed by ELISAs. \(*P < 0.01\)
compared with the untreated group; **$P<0.01$, compared with the NC siRNA-transfected group; ***$P<0.01$, compared with the cortactin siRNA-transfected group and the MARCK siRNA-transfected group.